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(57) Abstract

The present invention concerns a GEF mutant in which tryptophan (W) corresponding to position 1056 of the protein called $CDC25^{Mm}$ is mutated to an acidic amino acid, preferably glutammic acid, or tryptophan (W) at position 1056 and serine at position 1124 are mutated into an acidic acid and valine, respectively. The invention further provides the gene sequences encoding said amino acid sequences, and their application in the treatment of proliferative disorders and in the development of assays suitable for the identification of candidate agents able to disrupt the ras/GEF complex.

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MUTANTS OF GEF PROTEINS

Description

The present invention relates to an amino acid sequence of a Guanine Nucleotide Exchange Factor (GEF) which bears such mutation(s) to enable the effect of "sequestering" the Ras-protein effector molecule in an inactive state through a nucleotide-free ras/GEF-mutant complex. In particular the invention provides a mutant of a Guanine Nucleotide Exchange Factor (GEF) in which tryptophan (W) corresponding to position 1056 of the protein called $CDC25^{Mm}$ (Swiss-prot Entry P27671) is mutated to an acidic amino acid, or both tryptophan (W) at position 1056 and serine at position 1124 are contemporaneously mutated into an acidic amino acid and valine, respectively, resulting in a protein which slows down the GDP/GTP exchange because it does not catalitically dissociates ras from the nucleotide.

Another aspect of the invention concerns the gene sequence encoding said GEF-mutants.

The polypeptides/proteins of the invention take part in the activation cycle of proteins of the Ras family, as said, by "sequestering" them in the form of a complex mutant GEF/nucleotide-free Ras, so blocking the signal transduction pathway in which said Ras proteins are involved; this inhibitory action of the Ras cycle has applications in research, in the treatment of pathologies related to functional alterations of the Ras protein, like proliferative disorders, and in the development of assays suitable for the identification of agents able to disrupt the ras/GEF complex.

Background of the invention

It is known the pivotal role played by Ras proteins in the control of cell differentiation and cell proliferation.

In their action, they act as molecular switches cycling between an active GTP·bound state and an inactive GDP·bound state, because of a transit among a series of different conformational states. Following extracellular stimuli, the inactive GDP·bound-Ras protein, releases GDP attaining a transient "emtpy" state, which afterwards binds GTP thus reaching the active state. The intrinsic GTPase activity hydrolyzes GTP bringing Ras back to the inactive state. This cycle is unidirectional because the GTPase reaction is irreversible and because the intracellular GTP concentration is about 10 times higher than GDP concentration, so that is GTP that preferentially binds the "empty" state.

The above described Ras cycle, and thus the levels of active Ras protein, depend on the relative activities of two protein classes which are the targets of extracellular signals. GTPase Activating Proteins (GAP) stimulate intrinsic GTPase activity of Ras proteins while the GEF, "Guanine nucleotide Exchange Factors" catalyze the GDP/GTP exchange thus favouring the formation of the active Ras·GTP complex (see Fig. 1).

A series of experiments using both deletion and site-directed mutagenesis have identified some regions of interaction between the Ras proteins and the catalytic domain of exchange factors. In particular, regions within the Loop4/switch2 (aa 64-77) appear

important. The existance of a Ras/GEF intermediate has been shown both *in vivo* and *in vitro* and underlies the dominant negative effect played by ras proteins mutated in position 15, 16 and 17, which have a reduced affinity for nucleotides and sequester GEF in the form of inactive ras/GEF complexes (Polakis and Mc Cormick, 1993, J. Biol. Chem., 268, 9157-9160).

The first Ras-specific exchange factor to be cloned and sequenced was the product of the CDC25 gene of *Saccharomyces cerevisiae* (Camonis et al., *EMBO J* 5, 1986; Martegani et al., 1986 *EMBO J* 5, 2363).

Two classes of Ras-GEF have been so far identified in mammals: the p140 encoded by $CDC25^{Mm}$ (also called Ras-GRF) (Martegani et al., 1992 *EMBO J* 11, 2151-57; Shou et al., 1992 *Nature* 358, 351-354) and mammalian Sos (Botwell et al., 1992 Proc. Natl. Acad. Sci. USA 89, 6511-6515). $CDC25^{Mm}$ has been the first mammalian exchange factor cloned by using functional complementation of a *cdc25* yeast mutation (Martegani et al., 1992, *supra*). The complete cDNA encodes a 140 kDa protein expressed only in the central nervous system. Higly homologous proteins have been later identified in rat brain (Ras-GRF) (Shou et al., 1992 *supra*) and in human brain (human CDC25) (Park et al., Gene 1994; WO93/21314). This protein contains in its C-terminal region a 240 amino acid domain structurally and functionally homologous to the catalytic region of yeast CDC25. Both the full length 140 kDa protein and truncated forms spanning C-terminal regions are active in yeast where they can substitute for endogenous CDC25, moreover they are efficient exchange factors *in vitro*

both for human $p21^{ras}$ and yeast RAS2, while they are inactive on other Ras-like proteins (ral; rap, rac etc.). Both the full length p140 and the truncated forms are efficient activators of $p21^{ras}$ *in vivo* and potentially transforming.

The catalytic domains of $CDC25^{Mm}$-like and Sos-like GEF are extremely conserved with each other and with *S. cerevisiae* CDC25 both from a structural and functional point of view as shown by the ability of said mammalian GEF catalytic domains to complement *cdc25* mutation in *S. cerevisiae*.

Ras proteins, once switched to the active state in the GTP·bound form may interact *via* the L2 region with their target or effector. This leads to cascade activation of the "Mitogen Activated protein Kinases" (MAPK) or "Extracellular signal Regulated Kinases" (ERK) (Marshall CJ, 1995, *Cell* 80, 179-185; Burgering BMT and Bos JL, 1995, *TIBS* 20, 18-22). MAPK activated by dual threonine and tyrosine phosphorylation migrates in the nucleus where it can phosphorylate transcription factors inducing transcription of several genes, such as *fos*.

Summary of the invention

It has now been found that mutation af a highly conserved tryptophan residue within GEF catalytic domain, in the position corresponding to amino acid 1056 of $CDC25^{Mm}$, with an acidic amino acid, preferably glutamic acid, or the same mutation together with the mutation of serine in position 1124 of $CDC25^{Mm}$, with valine, "attenuate" ras-mediated signal transduction by binding to Ras proteins in a stable manner, thus "sequestering" them in the form of an inactive Ras/GEF

complex.

Such mutations have been found to be dominant negative, because of the fact that a so-mutated GEF protein irreversibly associate to ras. In the prior art, WO93/21314 describes a human GEF and its encoding nucleic acids. The authors report some biochemical properties of the human counterpart of $CDC25^{Mm}$, but no mention is made to the possibility of blocking Ras proteins in an inactive state by "sequestering" the complex ras/GEF-mutants. The present finding that mutant GEF molecules - the term "molecule" comprising both entire proteins or their peptidic fragments - display such effect, allows to put in practice targeted pharmacological interventions in pathological alterations in which the ras pathway is activated, such as neoplastic growth or neointima formation following angioplastic surgery.

Detailed description of the invention

With the aim of identifying amino acids possibly involved in the interaction between the catalytic region of $CDC25^{Mm}$ and Ras proteins, amino acids responsible of conferring to the molecule the ability to function as a guanine nucleotide exchange factor, a multiple alignment among the catalytic regions of 12 GEF from evolutionary distant organisms such as *Saccharomyces cerevisiae*, *Drosophila*, *Mus musculus* and *Homo sapiens* has been performed, and some mutations have been brought in GEF-sequences in order to assess the biological activity of the mutated derivatives.

Alanine substitution at position 1124 within the GEF catalytic domain was found to originate a "gain-of-

function" protein with higher thermostability and specific activity, whereas valine substitution originates a "loss-of-function" protein, which is more thermolabile than wild type and has little or no exchange activity.

The tryptophan corresponding to position 1056 of $CDC25^{Mm}$ has been mutated with amino acids having different chemico-physical properties: alanine (A), glutamic acid (E), phenylalanine (F), leucine (L) and lysine (K). Furthermore, also serine corresponding to residue 1124 of $CDC25^{Mm}$ has been mutated, together with the above mentioned Trp mutation. The so obtained mutants have been studied in different assays, wherein either full length proteins or fragments corresponding to the catalytic domain (which in most cases are C-terminal while in other - Sos proteins - are in the central part, Fig. 2) have been used.

Mutants have been obtained by conventional means, using site-directed mutagenesis followed by plasmid construction for the expression of mutant GEF in *E. coli*, in the yeast *S. cerevisiae* and in mammalian cells.

Their biological activity has been assayed using the obtained constructs in experiments of complementation of the temperature sensitive growth defect of the TC7 yeast strain (relevant phenotype *cdc25-1*^{ts}), which is able to grow at the permissive temperature of 24°C but not at the restrictive temperature of 37°C. Transformation of the TC7 *S. cerevisiae* strain (*MAT a ade lys trp ura3 cdc25-1*^{ts}, Martegani *et al.*, 1986 *supra*) have been performed by the method of Ito (Ito *et al.*, 1983 *J Bacteriol* 153, 163-

168).

Analysis of yeast complementation experiments confirms that only the substitution W1056F is able to restore, although less efficiently than the wild type molecule, functionality of the cdc25-1^{ts} strain, while all the other tested single mutants were not able to complement in the yeast assay. In case of double mutants, while the double mutant $CDC25^{Mm}$W1056E/S1124A was able to complement the *cdc25-I* mutation, the $CDC25^{Mm}$W1056E/S1124V was found to be inactive.

A further verification of mutant functionality has been conducted in mammalian cells by means of a *fos*-luciferase activity assay, according to which mammalian cells have been cotransfected with a plasmid expressing a mutant GEF and a *fos*-luciferase reporter plasmid whose expression is a function of ras activity. It is well-known that ras activation brings about transcriptional activation of cellular *fos* genes.

The ras activation state, and the exchanger activity as well, can thus be indirectly determined by assaying the activity of the enzyme luciferase which accumulates following transcription of the luciferase gene controlled by the *fos* promoter. Overexpression of the cataytic domain of $CDC25^{Mm}$ results in a significative increase of *fos*-luciferase activity in a model system where hamster (CHO) or mouse (NIH3T3) fibroblasts are cotransfected with plasmids expressing $CDC25^{Mm}_{976-1262}$ and a reporter *fos*-luciferase plasmid in which the luciferase gene is under the control of a fragment of the promoter of the human *fos* gene (-711/+42) (Zippel *et al.*, 1994, *Int J Oncol* 4, 175-179).

In the same model system, all the tested mutants reduce the signal transduction pathway downstream of the ras protein, when compared to the wild type protein.

The results of the in vitro exchange assays demonstrate the efficacy of the mutants of the invention to competitively block the wild protein exchanger's effects on Ras.

In other words, said mutants play a sequestering role on Ras protein, bringing it in an inactive state. Those mutants bind Ras in a non functional way, so blocking the signal transduction pathway downstream. The results of competition experiments suggest that GEF mutations allow to stabilize $p21^{ras}$ in its empty nucleotide-free state, i.e the WE mutant GEF causes dissociation of the Ras·GDP complex without promoting nucleotide exchange.

In vivo transfection experiments, in which NIH3T3 cells are stimulated by Platelet Derived Growth Factor (PDGF), confirm the sequestering effects of the mutants of the invention. It is well-known that PDGF-stimulation of NIH3T3 cells produces its effects through a $CDC25^{Mm}$-independent pathway (Zippel et al., Oncogene 12, 1996, 2697-2703). The $CDC25^{Mm}$WE mutant transfection in NIH3T3 makes the same cells not responsive to PDGF, being Ras unavailable to signal transduction, regardless of the pathway involved. Double mutants $CDC25^{Mm}$W1056E/S1122V displayed an even higher dominant-negative effect.

Furthermore, cell culture experiments demonstrates that $CDC25^{Mm}$WE mutants are able to completely inhibit fibroblast transformation originated by oncogenic k-*ras*

expression.

The possibility to block ras activity finds several application in the treatment of pathologies derived from a ras hyperactivation state.

In fact all oncogenic $p21^{ras}$ versions present point mutations in amino acids important for the binding to the guanine nucleotide that block ras in the active state (ras·GTP) or make its formation easier (Lowy DR and Willumsen BM, 1993 *Ann Rev Biochem* 62, 851-891).

In a particular cell type, one *ras* mutation can predispose to a particular type of tumor: for instance in a cell of the lung epithelium can predispose to an adenocarcinoma.

Literature data have recently highlighted the applicability of ras antagonist molecules in pathological situations different from tumors. In particular it has been shown how proliferation of VSMC (Vascular Smooth Muscle cells) induced by PDGF, FGF or thrombin is associated with ras induction and that VMSC with a dominant negative ras mutant display a significative reduction of proliferation induced from the same growth factors (Iran et al., *Biochem Biophys Res Comm* 202, 1252). The same ras mutant has been subsequently tested *in vivo* in a rat angioplastic model and a significative inhibition (60%) of neointima formation has been obtained 14 days after surgical operation (Indolfi *et al.*, 1996 *Nature Medicine* 1, 541-545). Moreover recent studies have shown how chemotactic cytokines directly induce ras (Knall et al., 1996, J Biol Chem 271, 2832) or molecules related to ras intracellular activity (Bokocl , 1995, *Blood* 86, 1649)

so extending the pharmacological field of action of specific ras antagonists towards biological effects non exclusively related to proliferation, such as cell motility.

Thus the dominant negative mutants of the invention can be effectively employed not only in the oncological field, but also in cardiovascular disorders, such as arterial restenosis following angioplastic therapy, or in the treatment of inflammatory states.

On the basis of what it has been said above, a first object of the invention relates to a GEF mutant in which the tryptophan (W) corresponding to position 1056 in the GEF protein called $CDC25^{Mm}$, is mutated to an acidic amino acid, or in which, besides the same substitution of the tryptophan, serine corresponding to position 1124 of $CDC25^{Mm}$ is mutated to valine.

The substitution of the tryptophan with glutamic acid is preferred.

The mutant's sequence could be extended to the whole molecule, to the catalytic domain or, more generally, to whatever part of the molecule, provided that, when tryptophan is the only amino acid mutated, a certain number of upstream and downstream amino acids with respect to mutated tryptophan are included, for a minimum of three amino acid upstream and three amino acid downstream of the mutated site, while, when both tryptophane and serine are mutated, at least the region comprising tryptophane and serine, and whichever flanking amino acid may be required to stabilize the mutant, is included, and provided that the peptide/protein is able to bind proteins of the Ras

family competing with native GEF proteins, preferably in a dominant-negative manner.

It will be possible to incorporate the mutant's amino acid sequence in a fusion protein or combined in such a way to obtain chimaeric proteins with the desired pharmacological properties. It will also be possible to chemically modify peptides in order to increase their *in vivo* stability and/or bioavailability.

Another object of the invention relates to the gene sequence encoding the above described protein or peptide, in which the codon corresponding to the Trp of the catalytic domain equivalent to position 1056 in $CDC25^{Mm}$ is substituted with a codon for an acidic amino acid, preferably glutamic acid, or the codons corresponding to Trp of $CDC25^{Mm}$ position 1056 and to Ser of $CDC25^{Mm}$ position 1124 are substituted, respectively, with a codon for an acidic amino acid, preferably glutamic acid, and a codon for valine; plasmids carrying said nucleic acid sequences are also comprised.

The invention further provides a method for screening substances useful to selectively disrupt the ras/GEF complex. Both in vivo and in vitro assays can thus be devised in order to screen for $p21^{ras}$ inhibiting molecules. By using the recently described inverse two hybrid technique (Vidal, M. Brachmann, R., Fattaey, A., Harlow, E., Boecke, J.D. 1996 Proc. Natl. Acad. Sci. USA 93, 10315-10320) molecules disrupting the GEF-mutants/ras interaction can be isolated by positively screening for fluororotic-resistant colonies. The screened molecules may include either cDNA and/or oligonucleotide libraries or (combinatorial) libraries

of chemical compounds. Another technique which may be used in screening compounds disrupting ras/GEF interaction is Scintillation Proximity Assay, SPA (EP0154734). The in vivo assay may comprise: a) providing a cell expressing the GEF-mutants either by themselves or as fusion proteins, whereby the expression of said mutants and/or their interaction with ras results in an easily scorable phenotype, b) contacting the cell with a candidate agent, c) measuring the scorable phenotype, d) comparing the scorable phenotype in the presence of the candidate agent to that of the untreated control.

Differently, the in vitro assay may comprise: a) providing GEF-mutants, either by themselves or as fusion proteins, whereby interaction of mutants with ras results in an easily scorable property, b) contacting the complex with a candidate agent, c) measuring the scorable property, d) comparing the scorable property in the presence of the candidate agent to that of the untreated control.

The mutants of invention or their derivatives can be used, as said, in the therapy of tumor forms, mainly due to ras activating mutations, cardiovascular diseases, such as arterial restenosis or inflammatory states.

For these purpouses peptides, proteins, nucleic acids or their derivatives will be delivered in adeguate pharmaceuticals compositions according to what is described, for instance, in "Remington's Pharmaceutical Sciences Handbook", Mack Publishing Company, New York USA.

Alternatively, it will be possible to deliver plasmids locally or, when necessary, gene therapy will be used, for instance using suitably modified retroviral vectors, carrying the above described gene sequences.

The compositions according to the invention will contain an effective quantity of mutant, variable as a function of the delivery route, of the pathology to be treated, of general patient conditions and will be preferentially delivered by parenteral route, in particular by intramuscular or subcutaneous injection.

Of course, also the daily dosage will be affected by several factors, such as pathology severity, weight, age and sex of the patient.

Other delivery routes are also possible, such as the oral route, by using formulation of the polypeptides in liposomes or other techniques known for polypetide or protein delivery by gastroenteric route, such as those described in WO93/25583.

Brief description of the drawings

Figure 1 shows a scheme of the Ras cycle,

Figure 2 shows a scheme of Ras-specific exchange factor of the Sos and CDC25-like family,

Figure 3 shows an assay of activation of a ras-dependent reporter gene in mammalian fibroblsts,

Figure 4 shows a standard exchange (a and c) and dissociation (b and d) assay *in vitro* on $p21^{ras}$ (a and b) and RAS21 (c and d) proteins for wild type GEF protein $CDC25^{Mm}_{976-1262}$ and the mutant GEF protein $CDC25^{Mm}_{976-1262}W1056E$, $CDC25^{Mm}_{976-1262}W1056A$, $CDC25^{Mm}_{976-1262}W1056L$, $CDC25^{Mm}_{976-1262}W1056F$,

Figure 5 shows that $CDC25^{Mm}WE$ is able to dissociate

non-catalytically ras-bound nucleotide, i.e.; only when present in equimolar amounts compared to $p21^{ras}$ or RAS2 proteins.

Figure 6 shows inhibition of the activity of a ras-dependent reporter gene (*Fos*-luciferase) by mutants $CDC25^{Mm}_{1-1262}WE$ as well as by double mutant $CDC25^{Mm}_{1-1262}W1056E/S1124V$. Data are average + standard deviation of three experiments performed on cells stimulated with PDGF, serum or nothing for 16 hours before assay.

Figure 7 shows the dimension of tumors formed in athymic *nu/nu* mice by 226.4.1 (transformation *focus* of NIH3T3 cells transfected with the k-*ras* gene) cells, G2.3. (226.4.1 cells cotransfected with control plasmid (pcDNA3); G2.DN.4 (226.4.1 cells transfected with mutant $CDC25^{Mm}_{1-1262}WE$) cells.

The following examples will clarify the invention:

Example 1

Construction of mutant GEF by site-directed mutagenesis

For all standard recombinant DNA manipulations, conventional procedures have been used unless otherwise indicated. A complete collection of such procedures is reported for instance in Sambrook *et al.*, (1989) Molecular Cloning.

Oligonucleotide assisted site-directed mutagenesis consists in hybridizing *in vitro* a single strand DNA with a synthetic oligonucleotide which is complementary to the single strand DNA except for a central mismatch region. In order to mutagenize the Trp1056 codon, the $CDC25^{Mm}$ 3'-terminal region of 1238 base pairs, presenting an elevated homology with proteins of the same family, has been cloned in an expression vector

called pALTER-1 (Promega), plasmid carrying a bacteriophage replication origin (M13 and R408) and two antibiotic resistance-encoding genes. One of these genes, encoding tetracyclin resistance is always functioning. The other, encoding ampicillin resistance, is instead inactive. After infection with R408 of an *E. coli* culture previously transformed with the above-mentioned construct, it is possible to obtain phage particles carrying single strand plasmid DNA (ssDNA). Mutagenesis is based on the use of two primers. One primer is able to recover the Ampicillin resistance, the other is designed with one or more mismatches necessary to introduce the desired amino acid substitution in the gene product of interest. After *in vitro* synthesis of the second DNA helix, it is transformed into an *E. coli* strain mutated in the DNA repair mechanism (BMH 71-18 *mutS*) so that it can mantain *in vivo* the mismatches introduced with the synthetic oligonucleotides. A second transformation cycle in strain JM109 allows a correct segregation of mutant and wild type plasmids ensuring a elevated proportion of plasmids with the mutated construct.

Further details can be found in the booklet accompanying the kit. Any other conventional method can be chosen to effect mutagenesis.

Mutagenized genes were completely sequenced with the dideoxy chain termination method so obtaining plasmids: pALTER-CDC25$^{Mm}_{976-1262}$W1056E, pALTER-CDC25$^{Mm}_{976-1262}$W1056A, pALTER-CDC25$^{Mm}_{976-1262}$W1056L, pALTER-CDC25$^{Mm}_{976-1262}$W1056F, pALTER-CDC25$^{Mm}_{976-1262}$W1056K.

Mutants were obtained by site-directed mutagenesis, using the following oligonucleotides:

MUTATION	OLIGONUCLEOTIDE
$CDC25^{Mm}_{976-1262}W1056A$	5'-TTGGCCAGGGCGCGATGAAGGCCGA-3'
$CDC25^{Mm}_{976-1262}W1056E$	5'-TTGGCCAGGGCGAGATGAAGGCCGA-3'
$CDC25^{Mm}_{976-1262}W1056F$	5'-TTGGCCAGGGCTTCATGAAGGCCGA-3'
$CDC25^{Mm}_{976-1262}W1056L$	5'-TTGGCCAGGGCTTGATGAAGGCCGA-3'
$CDC25^{Mm}_{976-1262}W1056K$	5'-TTGGCCAGGGCAAGATGAAGGCCGA-3'
$CDC25^{Mm}_{976-1262}$ S1124V	5-AGATCACCTCCGTCATCAACCGCAG-3
$CDC25^{Mm}_{976-1262}$ S1124A	5-AGATCACCTCCGCCATCAACCGCAG-3

$CDC25^{Mm}W1056E/S1124A$ and $CDC25^{Mm}W1056E/S1124V$ double mutants were constructed by swapping a 0.7 kbp *Nde*I/*Eco*RI restriction fragment encompassing codon 1124 between the $CDC25^{Mm}WE$ and $CDC25^{Mm}SA$ ($CDC25^{Mm}SV$) encoding genes.

Example 2

Construction of plasmids for expression of mutant GEFs in E. coli, in the yeast S. cerevisiae and in mammalian cells.

Inserts encoding $CDC25^{Mm}_{976-1262}$ from each derived plasmid or an appropriate fragment were subcloned in the appropriate expression plasmids as described below.

In order to construct plasmids for expression of the catalytic domain (residues $_{976-1262}$) of $CDC25^{Mm}$ in yeast and mammalian cells, the *Xba*I-*Xba*I 1.3 kbp fragment from each plasmid of the pALTER series was excised from said plasmids, the ends made blunt by treating with the Klenow fragment of *E. coli* DNA polymerase I. Plasmid pCYM-1 (Camonis et al., 1990, *Gene* 86, 263-268) is a shuttle vector in which expression of the inserted gene is controlled by the SV40 promoter,

which is functional both in the yeast *S. cerevisiae* and in higher eukaryotic cells. The pCYM-1 was linearized with the restriction enzyme *Bam*HI; the ends were made blunt by treating with the Klenow fragment of *E. coli* DNA polymerase I and subsequently dephosporylated with alkaline phosphatase. The wild type and mutant $CDC25^{Mm}$ fragments and linearized and dephosporylated pCYM-1 plasmid were ligated according to standard procedures. Insertion of each fragment in the proper orientation was checked by retriction and sequence analyses. The following plasmids were thus obtained: pCYM-$CDC25^{Mm}_{976-1262}$; pCYM-$CDC25^{Mm}_{976-1262}$W1056E; pCYM-$CDC25^{Mm}_{976-1262}$W1056A; pCYM-$CDC25^{Mm}_{976-1262}$W1056L; pCYM-$CDC25^{Mm}_{976-1262}$W1056F; pCYM-$CDC25^{Mm}_{976-1262}$W1056K, pCYM-$CDC25^{Mm}_{976-1262}$W1056E/S1124A; pCYM-$CDC25^{Mm}_{976-1262}$W1056E/S1124V.

The starting point for the construction of plasmids expressing in *E. coli* hybrid proteins between glutathione-S-transferase (GST) and $CDC25^{Mm}_{976-1262}$ was plasmid pGEX2T- $CDC25^{Mm}_{976-1262}$ (Martegani et al., 1992 supra). Such plasmid expresses a fusion protein between GST and the catalytic domain of $CDC25^{Mm}$ (residues 976-1262) under the control of an IPTG-inducible promoter. Plasmid pGEX2T-$CDC25^{Mm}_{976-1262}$ was cut with *Sph*I and *Hin*dIII, digestion releasing a fragment of about 440 bp spanning the Trp1056 codon. The plasmid was dephosphorylated with alkaline phosphatase and purified by preparative agarose gel electrophoresis. The *Sph*I-*Hin*dIII fragment spanning mutated Trp1056 codon was excised from each pALTER-based plasmid decribed in the previous paragraph and subcloned in said pGEX2T-$CDC25^{Mm}_{976-1262}$ plasmid cut with *Sph*I and *Hin*dIII,

dephosphorylated and purified by preparative electrophoresis. Insertion of each fragment in the proper orientation was checked by retriction and sequence analyses. The following plasmids were thus obtained: pGEX2T-CDC25^{Mm}$_{976-1262}$W1056E; pGEX2T-CDC25^{Mm}$_{976-1262}$W1056A; pGEX2T-CDC25^{Mm}$_{976-1262}$W1056L; pGEX2T-CDC25^{Mm}$_{976-1262}$W1056F; pGEX2T-CDC25^{Mm}$_{976-1262}$W1056K.

The *Nsi*I-*Dra*III fragment spanning the mutated 1056 codon obtained by digesting the appropriate pALTERCDC25^{Mm} plasmid was purified by preparative agarose gel electrophoresis and subcloned in plasmid pGEM3ESCDC25^{Mm} (carrying codons 812-1262 of CDC25^{Mm}) digested with the same restriction enzymes, so substituting the wild type fragment with the mutant one. The complete CDC25^{Mm} wild type or mutant gene was reconstructed by subcloning in the appropriate *Bam*HI and *Sac*I-cut pGEM3ESCDC25^{Mm} the *Bam*HI-*Sac*I fragment carrying codons 1-811 of CDC25^{Mm} which has been purified by agarose gel electrophoresis. In the constructs so obtained, the wild type or mutant CDC25^{Mm} gene is flancked by *Xba*I sites. The *Xba*I fragment carrying the full length CDC25^{Mm} gene was purified by preparative agarose gel electrophoresis and cloned in the XbaI site present in the polylinker of the plasmid pcDNA3 (Invitrogen). The following plasmids were thus obtained in this way: pcDNA3-CDC25^{Mm}$_{1-1262}$; pcDNA3-CDC25^{Mm}$_{1-1262}$W1056E; pcDNA3-CDC25^{Mm}$_{1-1262}$W1056A. In such plasmids CDC25^{Mm} expression is controlled by the CMV promoter (Citomegalovirus). A plasmid encoding the full length CDC25^{Mm}W1056E/S1124V double mutant in plasmid pCDNA3 was

then constructed essentially as described above.

Example 3

Mutant GEF: complementation assay in S. cerevisiae cdc25 mutants

Transformed cells were plated on minimal selective medium, with glucose as a carbon source. The transformant plates after an incubation at 24°C for 36 hours (permissive temperature) were shifted to restrictive temperature (36°C). In such conditions the mutant strain does not grow, while the mutant transformed with wild type $CDC25^{Mm}$ gives visible colonies 48-72 hours after the shift at the restrictive temperature.

Flasks used for yeast growth in liquid medium were incubated in a Dubnoff water bath with shacking. Growth on plates was done in humidified atmosphere incubators. For all methods regarding yeast not explicitely described, see Guthrie and Fink, *Methods in Enzimology* 194).

The ability of each mutant- including the double mutants $CDC25^{Mm}_{976-1262}{}^{W1056E/S1124V}$ and $CDC25^{Mm}_{976-1262}{}^{W1056E/S1124V}$ -to complement, at the restrictive temperature of 37°C, the *cdc25-1*^{ts} mutation was scored; results reported in Table 1 give the ratio between colonies grown at 37°C and those grown at 24°C. Average ± standard deviation from at least three independent experiments are reported.

Table 1 *Functional complementation of the S. cerevisiae cdc25 mutation by mutant GEF*

Protein	Ratio colonies (37°C/24°C)
pCYM-1	0.00 ± 0.00

$CDC25^{Mm}_{976-1262}$	Wild type	0.87 ± 0.20
$CDC25^{Mm}_{976-1262}$	W1056A	0.00 ± 0.00
$CDC25^{Mm}_{976-1262}$	W1056E	0.00 ± 0.00
$CDC25^{Mm}_{976-1262}$	W1056F	0.76 ± 0.13
$CDC25^{Mm}_{976-1262}$	W1056L	0.00 ± 0.00
$CDC25^{Mm}_{976-1262}$	W1056K	0.02 ± 0.00
$CDC25^{Mm}_{976-1262}$	W1056E/S1124V	0.00 ± 0.00
$CDC25^{Mm}_{9761262}$	W1056E/S1124A	0.88 ± 0.11

Example 4

Mutant GEF: fos-luciferase assay in CHO hamster fibroblasts

Luciferase activity was assayed using Promega "Luciferase Assay System^{R}", measuring light emission with a luminometer, in conditions where light emitted in a given time interval was a linear function of added extract.

DNA used for transfections was purified by Quiagen "Plasmid Maxi Kit". CHO cells were transfected by the Calcium Phosphate technique with constant DNA amounts (3 µg of pCYM-1 carrying wild type or mutant $CDC25^{Mm}$ catalytic region) and left 40 hours in the absence of serum and in the presence of selenium and transferrin. These factors are required, in the absence of serum, to ensure good cell adhesion. Luciferase activity of aliquots (10 µl) of cell extracts was measured with a luminometer and Relative Light Units (RLU) values so obtained were normalized to the protein content of each sample. Data were expressed as relative luciferase activity, taking as 1 the value obtained by the wild type as shown in Fig. 5.

Calcium Phosphate Procedure

Cells are inoculated in 55 or 21 cm^2 dishes at 17000 cells/cm^2 in DMEM (Dulbecco's Modified Eagle Medium) containing 4 mM glutamine, penicilline and streptomycin 100 U/ml and 10% serum (FCS, Fetal calf Serum for CHO or NBS, Newborn Calf Serum for NIH3T3) or at such a density to allow cells to reach 70-90% confluence the next day. The next day it is necessary to change the medium at least one hour before the transfection.

1. Prepare the transfection mix by adding in the following order:

sterile water	480 ml
2X HBS pH 6.95	500 ml
plasmid DNA	up to 10 µg

$CaCl_2$ 0.122 M, Hepes 0.61 mM (starting from $CaCl_2$ 2M, Hepes 10 mM)

For 21 cm^2 dishes quantitities of each component were halved.

2. transfection mixes are incubated 30 min at room temperature

3. Add 1 ml/dish of mix after vortex agitation and incubate 6 hours at 37°C.

4. Suck up the medium and add TBS 1x plus 15% glycerol for 90 seconds in order to increase transfection afficiency.

5. Wash twice with TBS 1x and add serum-free medium (starvation medium) composed by DMEM, sodium selenite 4 mg/ml and transferrin 10^{-8} M.

6. If required stimulate with PDGF 100 ng/ml for 16 hoirs.

7. Extract cell proteins according to Promega's

protocol using lysis buffer. Buffer compositions are reported below:

Used Buffers

A. HBS buffer:20 x stock

$Na_2HPO_4 \cdot 2H_2O$	21 mM
dextrose	120 mM
NaCl	2.7 M
KCl	100 mM
Hepes	400 mM

NBS 2x (pH 6.95) is prepared by dilution of the 20 x stock.

B. $CaCl_2$-Hepes

$CaCl_2$	2 mM	
Hepes	10 mM	pH 5.5

C. TBS buffer: 20 x stock

TRIS	500 mM	pH 7.4
NaCl	2.74 M	
KCl	10 mM	
$CaCl_2$	14 mM	
$MgCl_2 \cdot 2H_2O$	10 mM	

Autoclave and store at - 20°C

D. TBS 1 x (500 ml)

sterile H_2O	475 ml
TBS 20 x	25 ml
Na_2HPO_4 0.6 mM	from 0.5 M Na_2HPO_4 stock

E. Lysis buffer (PROMEGA)

TRIS-H_3PO_4	24 mM
CDTA	2 mM
DTT	2 mM
Glycerol	10%
Triton X-100	1%

Example 5

Expression in E. coli and purification of mutant GEF

Purification was carried out at 4°C. Purification of GST-$CDC25^{Mm}_{976-1262}$ fusion proteins is an affinity chromatography which utilizes the glutathion-sepharose resin (Sigma).

The protocol employed is very similar for the purification of wild type and mutant $CDC25^{Mm}$ proteins as well as for the purification of $p21^{ras}$ and RAS2 proteins, all as GST-fusion proteins. In the latter case 1 mM $MgCl_2$ and 10 mM GDP are added to all buffers. The major steps can be summarized as follows:

1. Inoculate the *E. coli* strain transformed with the desired plasmid in LB plus ampicillin 100 µg/ml. Leave at 37°C over/night.
2. The next day 8 ml of preinoculum are diluted in 500 ml LB + ampicillin medium till the optical density at 600 nm of the bacterial culture reaches 0.4-0.6 OD.
3. Protein production is induced with 0.2 mM IPTG (Isopropyl-β-D-Thiogalactopyranoside) for 16 hours at 24°C (4 hours at 28°C for Ras proteins) when cell cultures reach a cell density of 1.5-1.9 OD.
4. Cells are collected by centrifugation at 8000 rpm for 5 minutes.
5. Dissolve cell pellet in 8 ml of lysis buffer made as follows: PBS 1x (NaCl 150 mM, Na_2HPO_4 16 mM, NaH_2PO_4 4 mMpH 7.3), β-mercaptoethanol 14 mM (7 mM for Ras proteins), EDTA1 mM, PMSF0.5 mM, 0.5% Triton X-100.
6. Cells are broken at 4°C using a French Press which

allows complete breackage of the cells.

7. 0.5 mM PMSF, 1% Triton X-100 and 50 Dnase U are added.
8. Centrifuge 20 minutes at 15000 rpm at 4°C.
9. Supernatant is recovered and incubate with 50% resin (previously hydrated with distilled water for at least one hour and washed three times with PBS 1x) for 50 minutes at 4°C with mild agitation.
10. Centrifuge 2 min at 1500 rpm at 4°C and discard supernatant.
11. Wash the resin twice with 10 ml PBS, 14 mM β-mercaptoethanol (7 mM for Ras proteins), 1 mM EDTA, 0.5% Triton X-100 (Buffer A, pH 8.5) and twice with 50 mM TRIS-HCl pH 8.5, 50 mM NaCl, 14 mM β-mercaptoethanol (7 mM for Ras proteins), 1 mM EDTA, 0.5% Triton X-100.
12. Incubate the resin three times with 4 ml Buffer A pH 8.5 for 15 minutes at 4°C in the presence of 3 mg/ml reduced glutathion.
13. Dialyze against 500 ml of 50 mM TRIS-Cl pH 7.5, 50 mM NaCl, 14 mM β-mercaptoethanol to concentrate ca. three times the protein.
14. Check on 10% SDS-polyacrilamide gels the different purification steps.

Ras proteins and, when required, wild type and mutant $CDC25^{Mm}$ proteins were separated from GST by thrombin cleavage as follows.

After last wash, before elution, resin is resuspended in Thrombin buffer (TRIS-Cl 50 mM pH 7.5, NaCl 50 mM, $CaCl_2$ 5 mM), the resin is centrifuged and resuspended in equal volume of Thrombin buffer

containing about 10 thrombin units/mg of fusion protein bound. After a ca. two hours at 4°C with mild shacking, the resin is then centrifuged and the soluble fraction, containing the protein of interest, is collected. The resin is washed twice with PBSand the three soluble fractions collected are pooled. A further protein purification step uses an ionic exchange columnn to eliminate thrombin residues and possible contaminating proteins. A Pharmacia MonoQ column with a with a 0-1M NaCl elution gradient are used; protein gets eluted at ca. 140 mM NaCl. protein-containing fractions are controlled by SDS-PAGE and pooled, if required are concentrated with Centripep 10 (Centricon) and dyalized over/night against 1 liter of TRIS-Cl 50 mM pH 7.5, NaCl 50 mM, glycerol 50%, β-mercaptoethanol 7 mM.

Proteins purified int this way were > 90% pure.

Example 6

Guanine nucleotide exchange and dissociation assays on $p21^{ras}$ and RAS2 proteins with GEF mutants

The use of labelled nucleotides allows to measure dissociation rates of Ras·GDP complexes, as well as the GDP/GTP exchange reaction by means of filtration on nitrocellulose filters (Millipore, 0.45 µm). Only Ras·bound nucleotides are retained on the membrane and radioactivity determination allows to measure the amount of complex retained on the membrane. Nitrocellulose membranes, soaked in the same buffer used in the reaction, are put on a HOFFER filtration apparatus connected to a vacuum pump which allows aspiration with a pression of 0.9 bar. After an incubation period at 30°C aliquots of the reaction are withdrawn and filtered

on the membrane.

Nitrocellulose filters are later air dried and counted in scintillation vials containing 5 ml of scintillation fluid (Ultima Gold Packard) and counted with a Prias Counter.

Dissociation reaction

Dissociation kinetics of the Ras·guanine nucleotide complexes have been studied charging p21 (or RAS2) protein with [^{3}H]GDP; dissociation of the complexes so obtained is followed as a function of time after adding an excess unlabelled nucleotide.

The $p21^{ras}$ or RAS2 protein (2.5 mM) is incubated in the presence of buffer A (50 mM TRIS-HCl pH 7.5, $MgCl_2$ 1 mM, 10 mM NH_4Cl, 0.5 mg/ml BSA), 3 mM EDTA and 15 µM [^{3}H]GDP.

After 5 minutes the reaction is stopped by adding 4 mM $MgCl_2$ and putting the tube in ice.

Dissociation rate of the labelled complex is measured after adding a 500 fold excess unlabelled nucleotide.

25 µl of said reaction mix are incubated at 30°C in buffer A containing 1.6 mM unlabelled GTP (GDP) in the presence or absence of different concentrations o wild type or mutant $CDC25^{Mm}$. Final volume of this raection is 120 µl; at predetermined times 25 µl aliquots are taken and the decrease in Ras·bound radioactivity is followed after nitrocellulose filtration as a function of time.

Exchange reaction

The exchange reaction is performed by incubating the p21·GDP or RAS2·GDP complex in buffer A in the

presence of [^{3}H]GTP (6 µM), in the presence as required of different concentrations of CDC25^{Mm}. Final volume is 120 µl. The increase in Ras·bound radioactivity (filter retained) corresponds to the exchange of unlabelled GDP with labelled GTP.

EDTA at a final 2 mM concentration is used as a positive control of the exchange reaction, because by chelating Mg^{++}, it greatly accelerates the GDP/GTP exchange reaction, so giving the maximum atteinable Ras·bound radioactivity. Results reported in Figure 4 show that under the assay conditions, none of the mutants is able to significativly stimulate exchange and dissociation of guanine nucleotides on both p21^{ras} and RAS2.

Example 7

Competition assay between mutant and wild type GEF

In order to understand whether the inactive mutants are still able to bind p21^{ras}, it has been evaluated if the mutant proteins were able to compete with the wild type exchanger in a typical exchange reaction. Wild type CDC25^{Mm} was thus incubated in the presence of a 10 fold molar excess of mutant GEF, p21^{ras}·GDP complex and radioactive GDP. As in previous experiments the increase in substrate·bound radioactivity was then followed as a function of time.

Adding a 10 fold molar excess of mutant protein CDC25$^{Mm}_{976-1262}$W1056F, CDC25$^{Mm}_{976-1262}$W1056A and CDC25$^{Mm}_{976-1262}$W1056L resulted in an exchange reaction whose rate was intermediate betwenn that of the uncatalyzed reaction and that catalyzed by the wild type exchanger.very similar to the control without exchanger.

After 30 minutes the reaction was inhibited between 30 and 50%, taking as 100% the difference between radioactivity reached by p21 in the presence of the wild type exchanger and that reached because of $p21^{ras}$ intrinsic exchange rate (Table 2). This obsevation indicates the these proteins still mantain the ability to interact with $p21^{ras}$ and thus the reason for their failure to function both *in vivo* and *in vitro* has to be found in stages following the ras/exchanger binding.

Of particular interest is the result obtained with mutant $CDC25^{Mm}_{976-1262}W1056E$. After 30 minutes p21·associated radioactivity is markedly lower than that determined by intrinsic substrate activity.

Table 2 Inhibition values by mutant GEF of the exchange reaction on $p21^{ras}$

Mutation	Inhibition after 30 minutes
$CDC25^{Mm}_{976-1262}W1056A$	47.2%
$CDC25^{Mm}_{976-1262}W1056E$	124.0%
$CDC25^{Mm}_{976-1262}W1056F$	49.1%
$CDC25^{Mm}_{976-1262}W1056L$	34.1%

Example 8

Induction of non-catalytic dissociation of Ras·bound nucleotides, without nucleotide exchange induction by mutant GEF

On the basis of said competition experiments it was hypothesised that mutant $CDC25^{Mm}_{976-1262}W1056E$ stabilizes the $p21^{ras}$ protein in its empty, nucleotide-free form. Such hypothesis holds that, when present at equimolar concentration with Ras·GDP, $CDC25^{Mm}_{976-1262}W1056E$ has a strong dissociating activity on the Ras·GDP complex, while being unable to promote exchange.

Such hypothesis has been confirmed by data presented in Fig. 5 A and B.

These data were confirmed and extended by data reported in Fig. 5C and D showing that:

- Wild type $CDC25^{Mm}_{976-1262}$ (200 nM or 400 nM) is able to displace the mutant exchanger $CDC25^{Mm}_{976-1262}W1056A$ (lines with crosses and asterisks in Fig. 5C) but not mutant $CDC25^{Mm}_{976-1262}W1056E$ (lines with crosses and asterisks in Fig. 5D).
- Adding $CDC25^{Mm}_{976-1262}W1056A$ to an exchange reaction containing wild type $CDC25^{Mm}_{976-1262}$ (closed circles, Fig. 5C) results in a modest variation in exchange rate. Adding mutant exchanger $CDC25^{Mm}_{976-1262}W1056E$ (closed circles, Fig. 5D) results in the time stable drop of substrate incorporated radioactivity provoked by wild type $CDC25^{Mm}$ down to values similar to those reached by the mutated exchanger alone. We chose to compare the effects of W1056A and W1056E mutations, since them map to the same amino acid position but differ in the introduced amino acid and in their ability to compete *in vitro* for substrate.

Example 9

Inhibition of expression of ras-dependent genes by mutant GEF in mammalian fibroblasts

In order to show whether $CDC25^{Mm}W1056E$ was able to display an inhibitory effect on ras *in vivo*, NIH3T3 cells were transfected with plasmids expressing the full length -more stable - form of $CDC25^{Mm}W1056E$ and $CDC25^{Mm}W1056A$ and stimulated with PDGF, 16 hours before assaying luciferase activity. PDGF stimulation allows to

activate ras in a $CDC25^{Mm}$-independent way, thus reaching elevated luciferase activity values, mandatory prerequisite to show by using transient transfections the presence of a dominant negative effect (Sakaue *et al.*, *Mol Cell Biol* 15, 379-388; Zippel *et al.*, 1996 *supra*).

In such conditions in cells transfected with $CDC25^{Mm}$W1056E protein the *fos* promoter is activated to levels significantly lower in comparison to cells transfected with the empty vector. These results thus indicate that the $CDC25^{Mm}$W1056E protein is able to attenuate the ras signal transduction pathway (Fig. 6).

The double mutant $CDC25^{Mm}$W1056E/S1124V strongly inhibits serum-stimulated expression of the *Fos*-luciferase promoter gene, under conditions in which the single $CDC25^{Mm}$WE mutant is less effective, indicating that the S1124V mutation reinforces the dominant negative nature of $CDC25^{Mm}$WE.

Example 10

Biological activity assay: $CDC25^{Mm}$W1056E mutant induces flat reversion of ras-transformed mammalian fibroblasts.

In order to evaluate the biological effects of $CDC25^{Mm}$W1056E mutant expression in mammalian cells, the plasmid carrying the mutant cDNA was stably transfected in murine NIH3T3 fibroblasts transformed with oncogenic ras and a morphological analysis of the transfected cells was performed. In fact one of the most evident effects of ras oncogene activation is cel transformation characterized by morphological alterations, so that a possible ras inhibition results in a regression of the transformed phenotype.

Cells used for this purpouse have been previously transformed by insertion of genomic human DNA carrying *k-ras* and selected on the basis of the ability to form *foci*. One of these *foci* (226.4.1) has been isolated, expanded and grown indefinitely. The morphology of such focus, constant over time, was thus typical of transformed cells: compared to parental NIH3T3, 226.4.1 had a rounder cell body, with well visible prolongations, displayed less substrate adhesion, and because of the loss of contact inhibition displayed disorganized and multilayered growth. 226.4.1 cells were thus transfected with the calcium phosphate method (fully illustrated in example 5) using the following constructs:

1. empty pcDNA3 control
2. pcDNA3 carrying full length $CDC25^{Mm}W1056E$

Following transfection geneticin resistent colonies were selected (plasmid pcDNA3 carries the gene encoding resistance to such antibiotic) and isolated after about 15 days of selection. Seven colonies were obtained with the control plasmid and eight with the mutant plasmid. Each colony has been expanded and analyzed by optical microscopy. Table 3 summarizes morphological features of the obtained colonies. While 7/7 of the control colonies displayed the typical transformed morphology, 7/8 of the colonies transfected with the mutant GEF presented a more or less pronounced reversion of the transformed phenotype.

In conclusion these results indicate that transfection of mutant $CDC25^{Mm}WE$ is able to inhibit ras-mediated cell transformation in mammalian fibroblasts.

Table 3 Effect of CDC25^{Mm}WE on morphology of transformed fibroblasts. Parameters evaluated to define transformed morphology were: loss of adhesion ability, loss of flat aspect, loss of contact inhibition. Transformation was scored on a scale from NIH3T3 untransformed cells, minimum level (-) and parental 226.4.1, maximum level (+++).

226.4.1-DERIVED CELL LINE	TRANSFECTED PLASMID	TRANSFORMED MORPHOLOGY
G2.1	Empty pcDNA3	+++
G2.2	Empty pcDNA3	+++
G2.3	Empty pcDNA3	+++
G2.4	Empty pcDNA3	+++
G2.5	Empty pcDNA3	+++
G2.6	Empty pcDNA3	++
G2.7	Empty pcDNA3	++
G2.DN.1	PCDNA3/CDC25^{Mm}W1056WE	-
G2.DN.2	PCDNA3/CDC25^{Mm}W1056WE	-
G2.DN.3	PCDNA3/CDC25^{Mm}W1056WE	-
G2.DN.4	PCDNA3/CDC25^{Mm}W1056WE	+/-
G2.DN.5	PCDNA3/CDC25^{Mm}W1056WE	+/-
G2.DN.6	PCDNA3/CDC25^{Mm}W1056WE	-
G2.DN.7	PCDNA3/CDC25^{Mm}W1056WE	++
G2.DN.8	PCDNA3/CDC25^{Mm}W1056WE	-

Example 11

Biological activity assay: CDC25^{Mm}W1056E mutant induces a severe delay in growth of ras-derived xenotransplants in nude mice

In order to further evalutate the biological effects of CDC25^{Mm}W1056E mutant expression in mammalian cells, the ability of representative stable transformed

cells described in the previous example to form tumor when injected in nude mice was tested. In more detail either $5*10^4$ or 10^5 cells were subcutanously injected in nude mice, and the size of the tumor measured over 40 days. Figure 7 shows the results obtained for the following clones:

226-4.1 k-*ras* transformed NIH3T3

G2.3 226-4.1 + empty pCDNA3

G2.DN.4 226-4.1 + pCDNA3CDC25^{Mm}WE

it is apparent that k-*ras* transformed cells expressing CDC25^{Mm}WE have a significative delay in tumor formation, since after 14 days when the average size of the tumor formed by 10^5 226-4.1 and G2.3 cells is 12 and 9 mm, respectively, no tumor can be detected. Eventually after a further 20 days, tumor starts growing also in G2.DN4 cells, possibly because of the appearance of cells whose cell cycle progression no longer requires ras activation.

In conclusion these results indicate that expression of mutant GEF CDC25^{Mm}WE is able to inhibit ras-mediated tumor formation in xenotransplants.

CLAIMS

1. Guanine nucleotide exchange factor (GEF)-mutants characterized in that they irreversibly block ras proteins in an inactive state through a nucleotide-free ras/GEF-mutant complex.

2. GEF-mutants according to claim 1, in which the Trp corresponding to position 1056 of $CDC25^{Mm}$ is mutated to an acidic amino acid.

3. GEF-mutants according to claim 2, in which said acidic amino acid is glutamic acid.

4. GEF-mutants according to claims 2-3, which comprise a number of amino acids sufficient to bind ras proteins in competition with native GEF-proteins.

5. GEF-mutants according to claim 4, which correspond to the mutated $CDC25^{Mm}W1056E$.

6. GEF-mutants according to claim 4, which correspond to the mutated $CDC25^{Mm}{}_{976-1262}W1056E$.

7. GEF-mutants according to claim 1, in which Trp corresponding to position 1056 of $CDC25^{Mm}$ is mutated into an acidic amino acid and Ser corresponding to position 1124 of $CDC25^{Mm}$ is mutated into valine.

8. GEF-mutants according to claim 7, in which said acidic amino acid is glutamic acid.

9. GEF-mutants according to claims 7-8, which correspond to mutated $CDC25^{Mm}W1056E/S1124V$.

10. GEF-mutants according to claim 7-8, which correspond to mutated $CDC25^{Mm}{}_{976-1262}W1056E/S1124V$.

11. Chimaeric proteins which can be obtained by combining the sequence of a GEF-mutant of any of the previous claims with the sequence of other proteins.

12. Gene sequence encoding for a GEF-mutant of claims 1-10.

13. Plasmid carrying the gene sequence of claim 12.

14. Pharmaceutical compositions containing as the active ingredient a GEF-mutant of claims 1-10.

15. Use of the GEF-mutants of claims 1-10 as medicaments.

16. Use of the GEF-mutants of claims 1-10 for the preparation of medicaments for the treatment of pathologies related to ras activation.

17. Use of the GEF-mutants according to claim 16 for the preparation of medicaments for the treatment of tumors, cardiovascular diseases, arterial restenosis, inflammatory states.

18. Use of the GEF-mutants of claims 1-10 as reagents in a screening assay of compounds able to dissociate the ras/GEF complex.

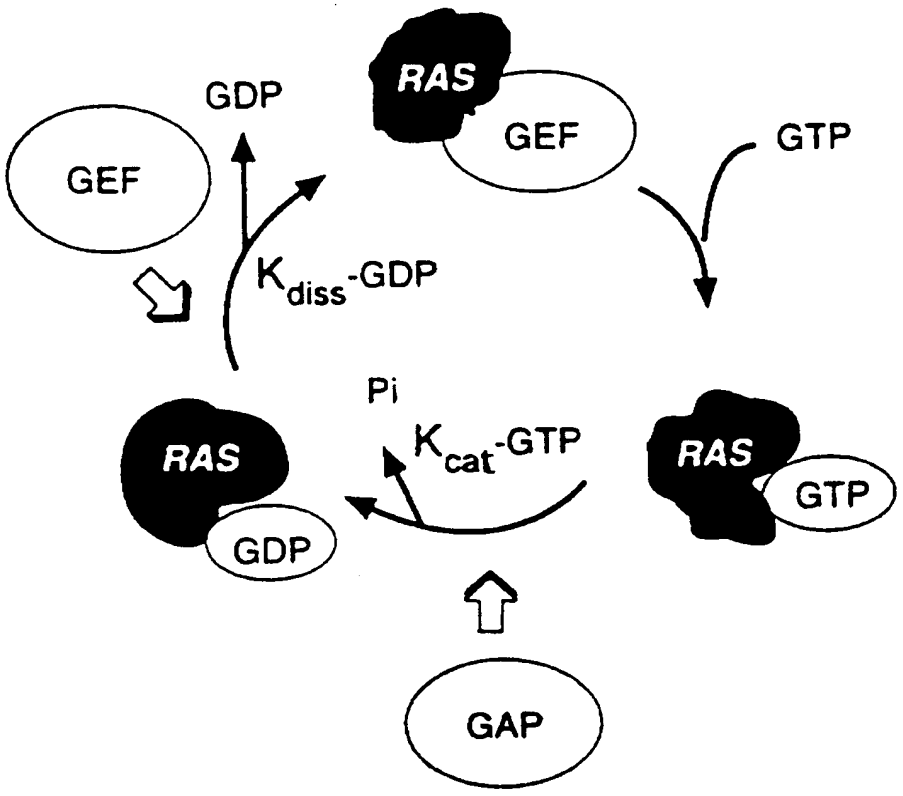


FIGURE 1

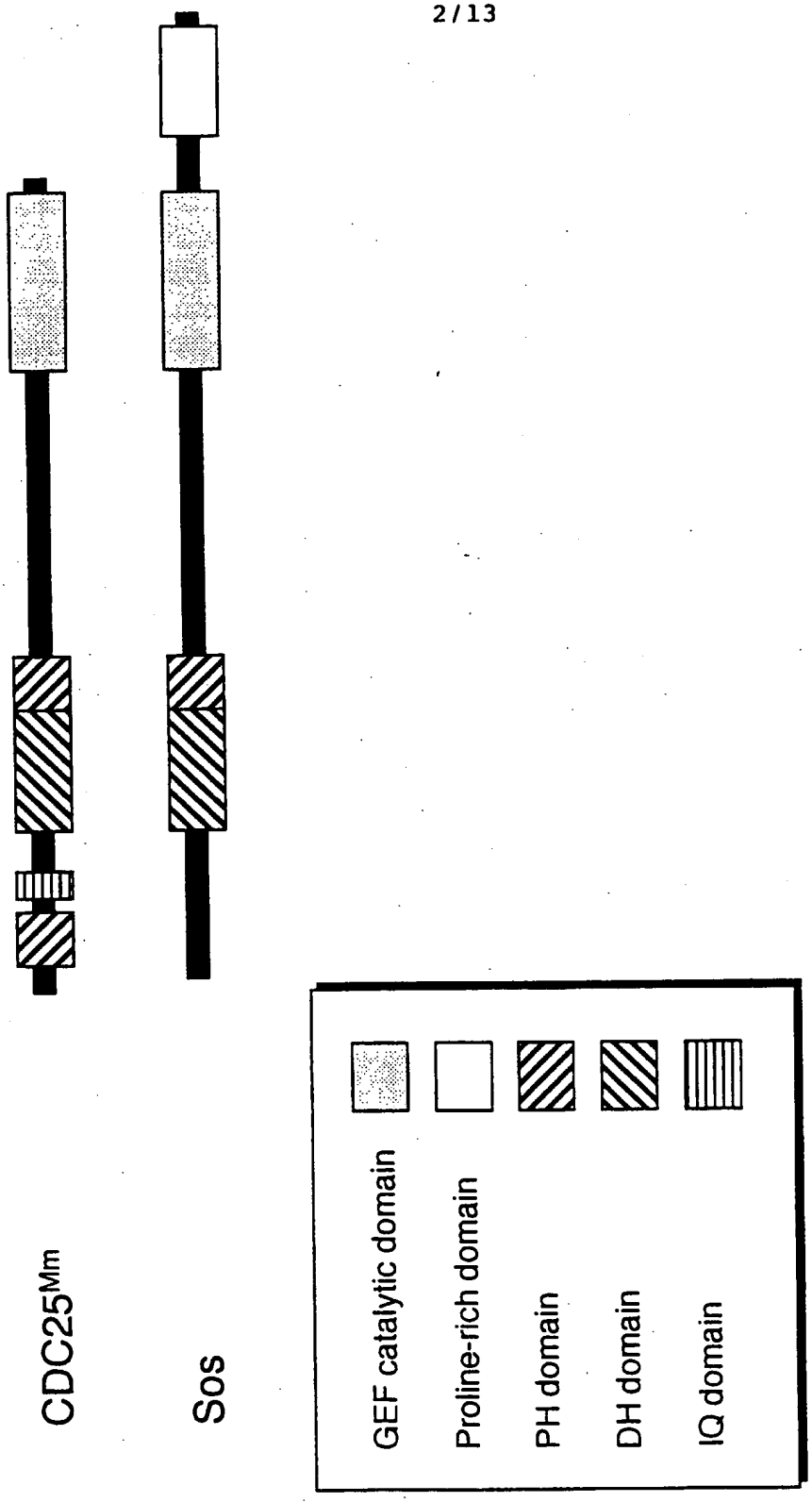

CDC25Mm
Sos
GEF catalytic domain
Proline-rich domain
PH domain
DH domain
IQ domain

FIGURE 2

FIGURE 3

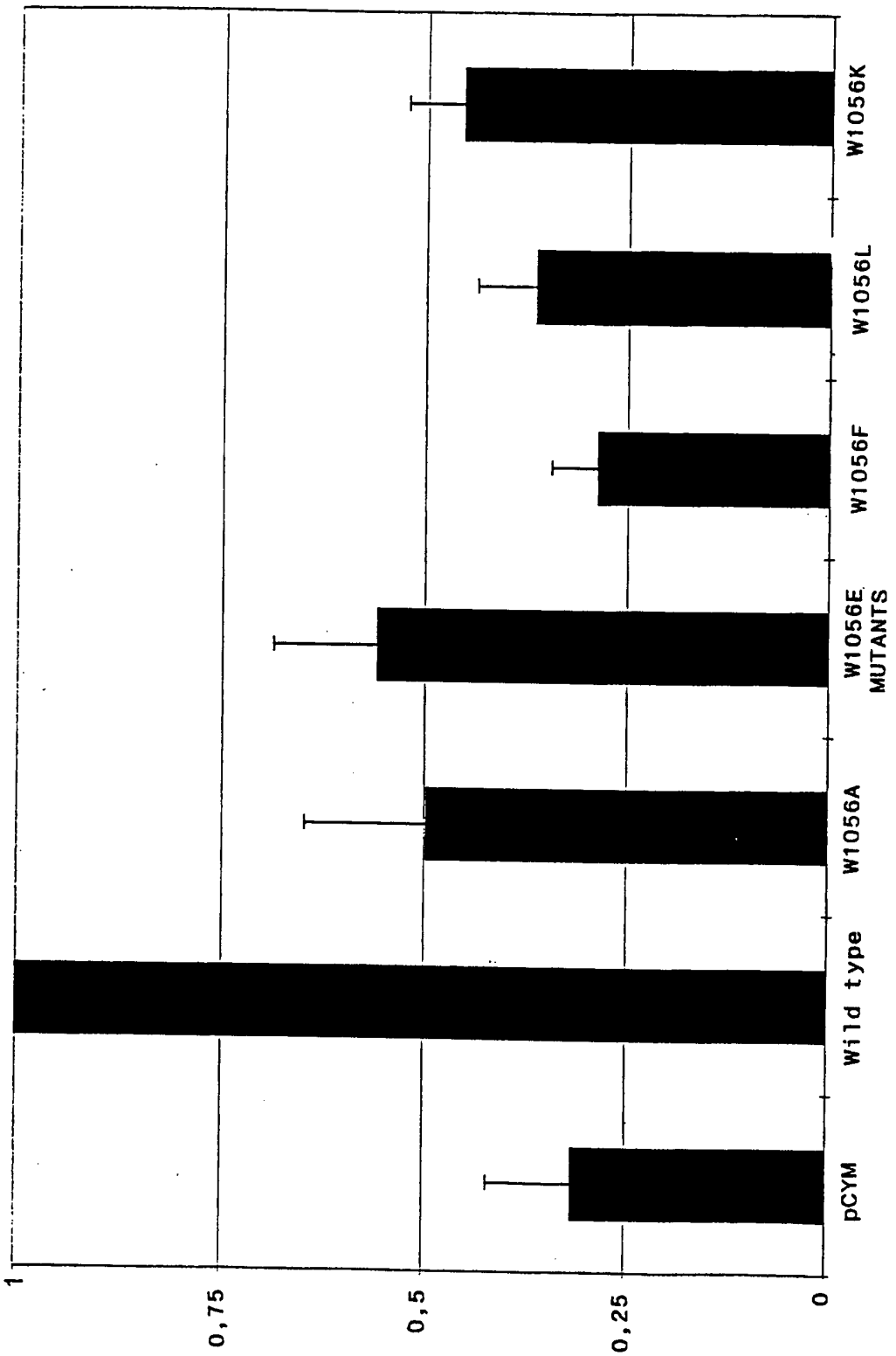

W1056K
W1056L
W1056F
W1056E MUTANTS
W1056A
Wild type
pCYM
0
0,25
0,5
0,75
1
RELATIVE LUCIFERASE ACTIVITY

FIGURE 4A

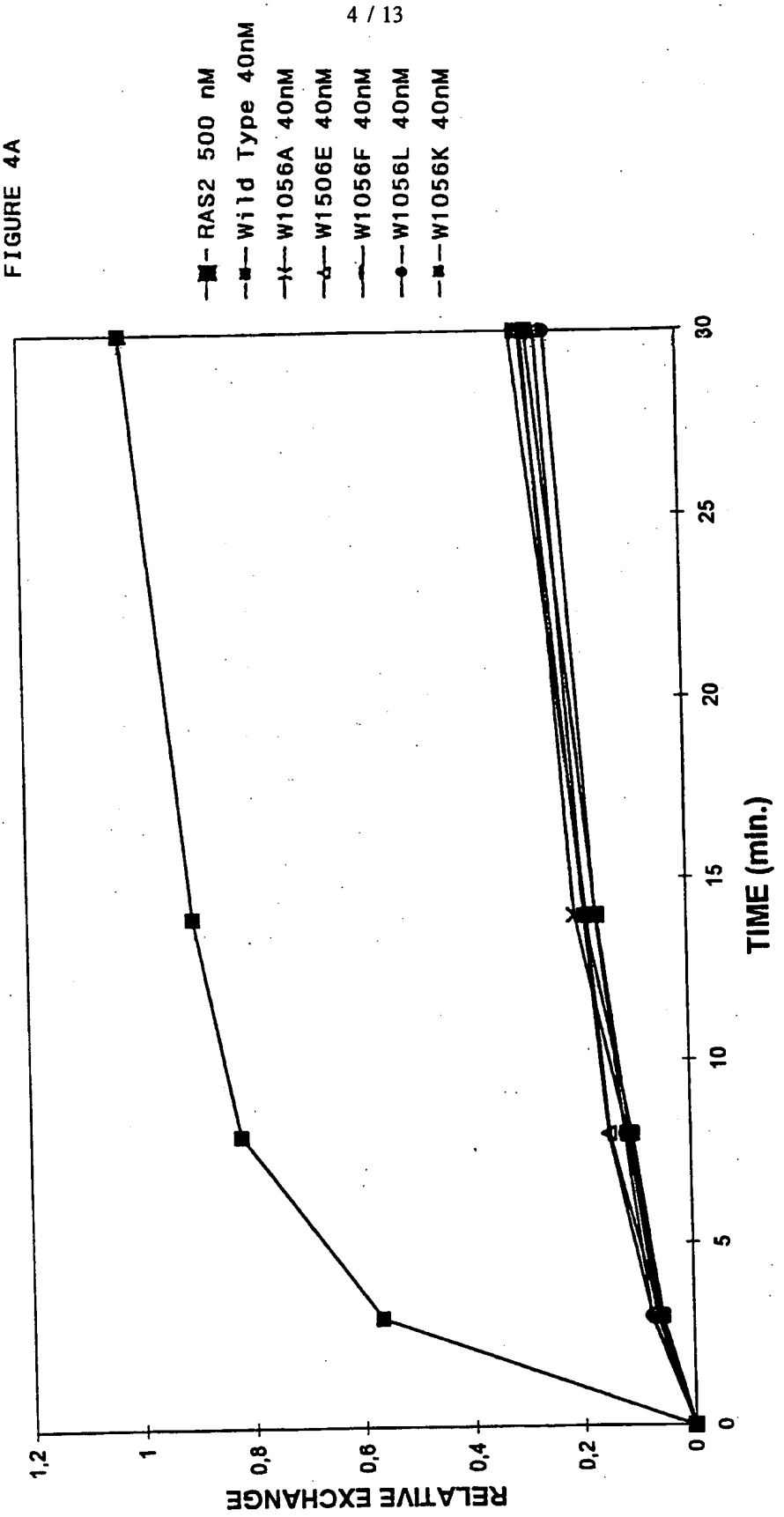

RAS2 500 nM
Wild Type 40nM
W1056A 40nM
W1506E 40nM
W1056F 40nM
W1056L 40nM
W1056K 40nM
RELATIVE EXCHANGE
TIME (min.)
0
0,2
0,4
0,6
0,8
1
1,2
0
5
10
15
20
25
30

FIGURE 4B

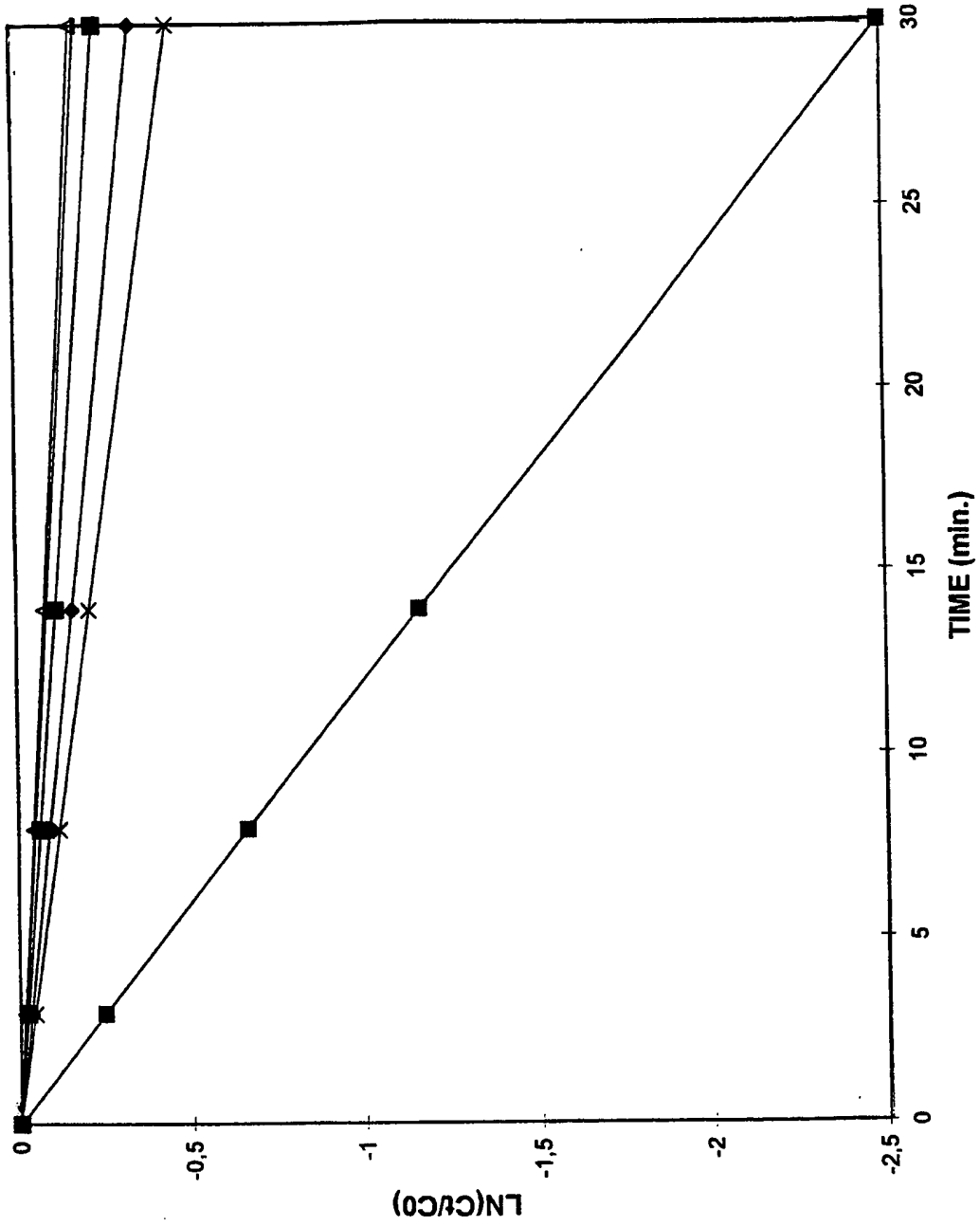


FIGURE 4C

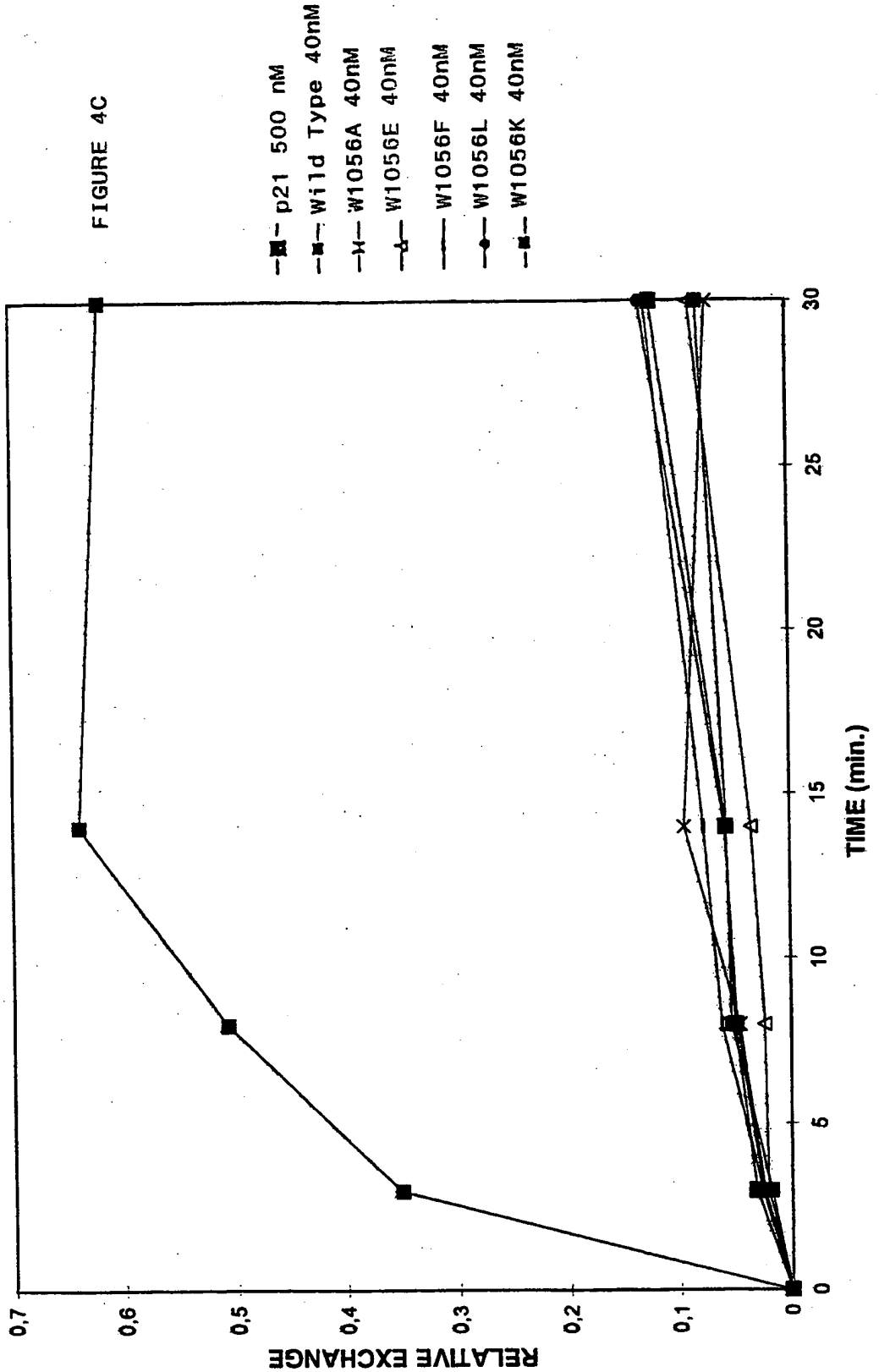
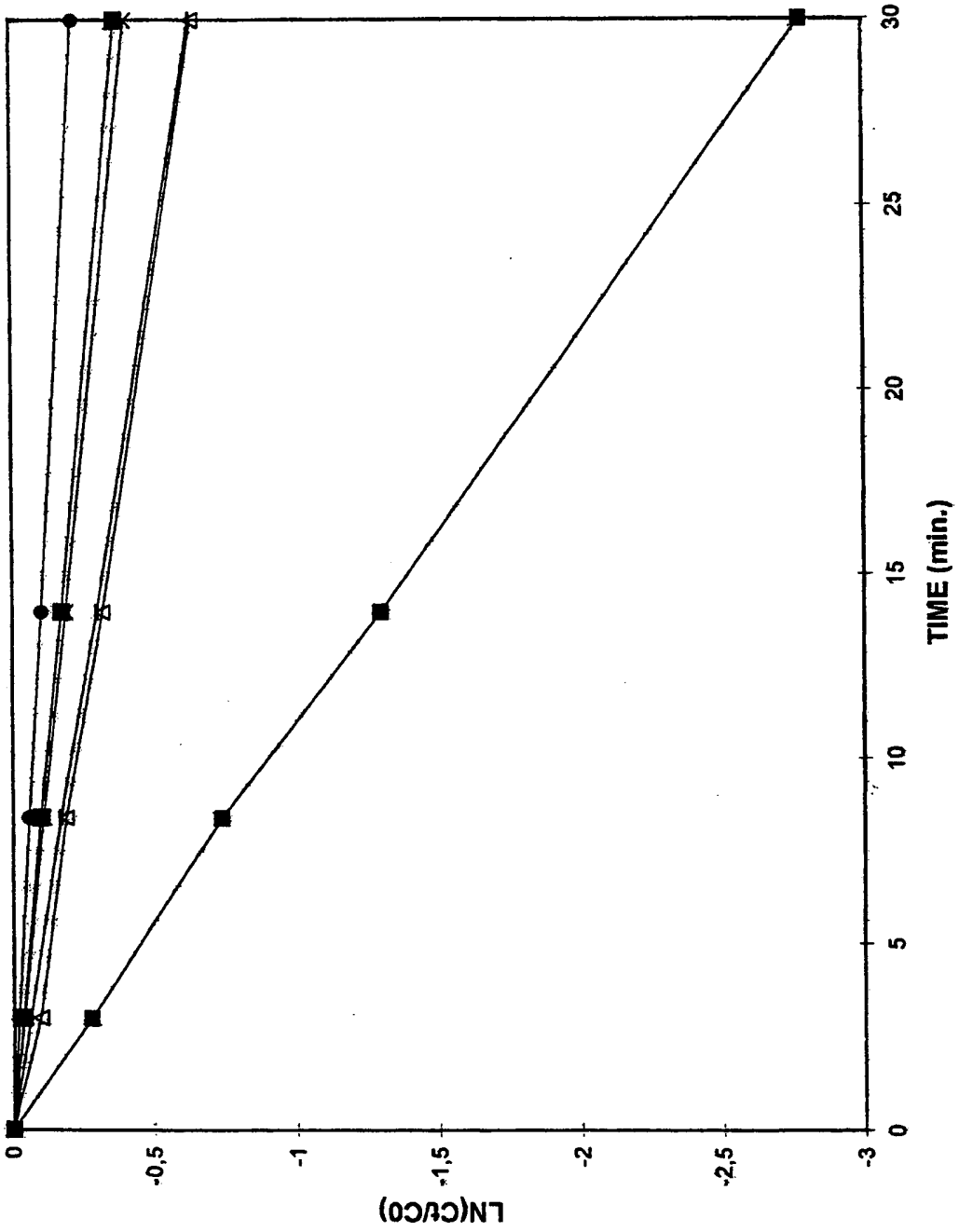

p21 500 nM
Wild Type 40nM
W1056A 40nM
W1056E 40nM
W1056F 40nM
W1056L 40nM
W1056K 40nM
RELATIVE EXCHANGE
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
0
5
10
15
20
25
30
TIME (min.)

FIGURE 4D



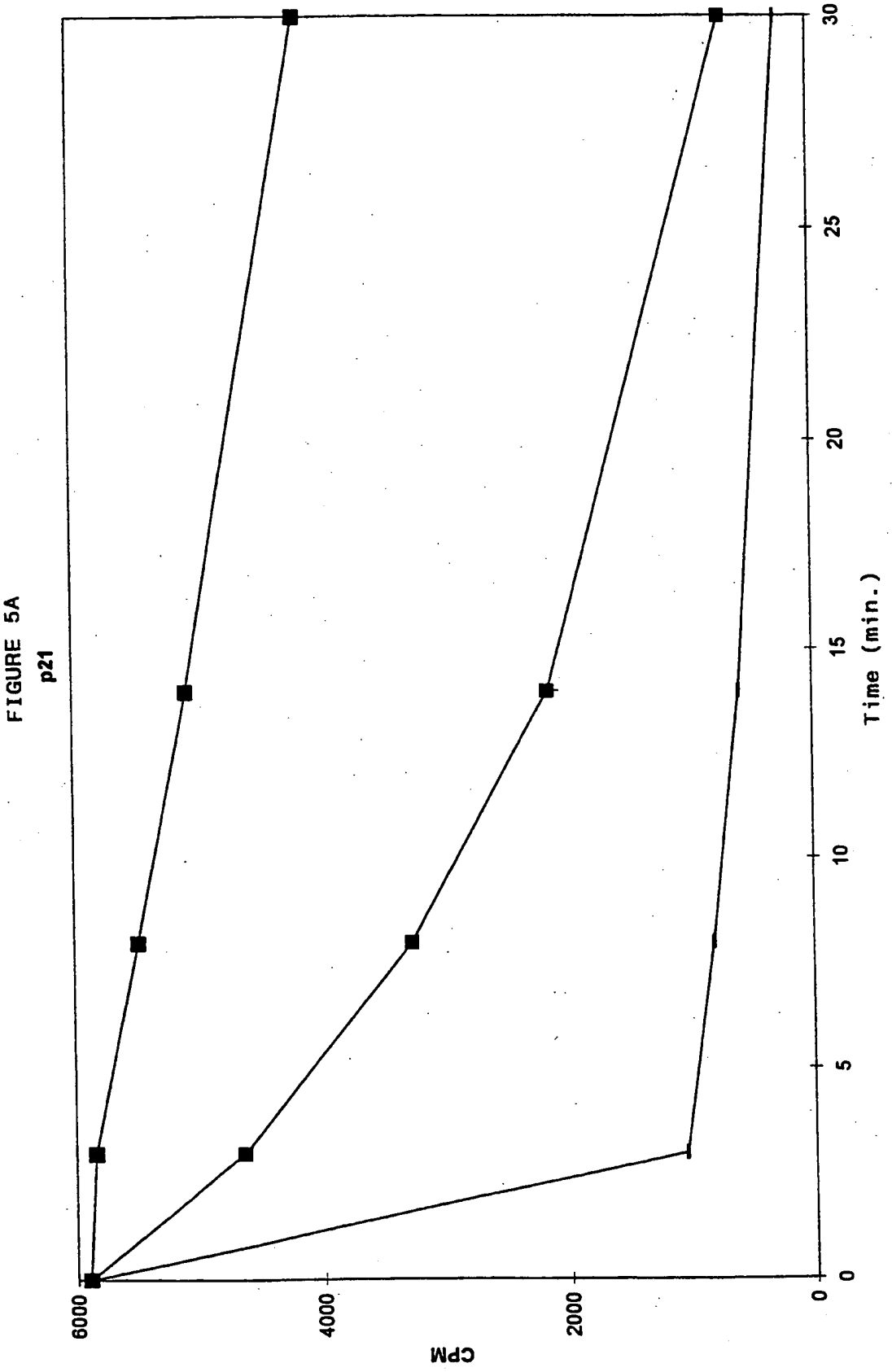


FIGURE 5A
p21

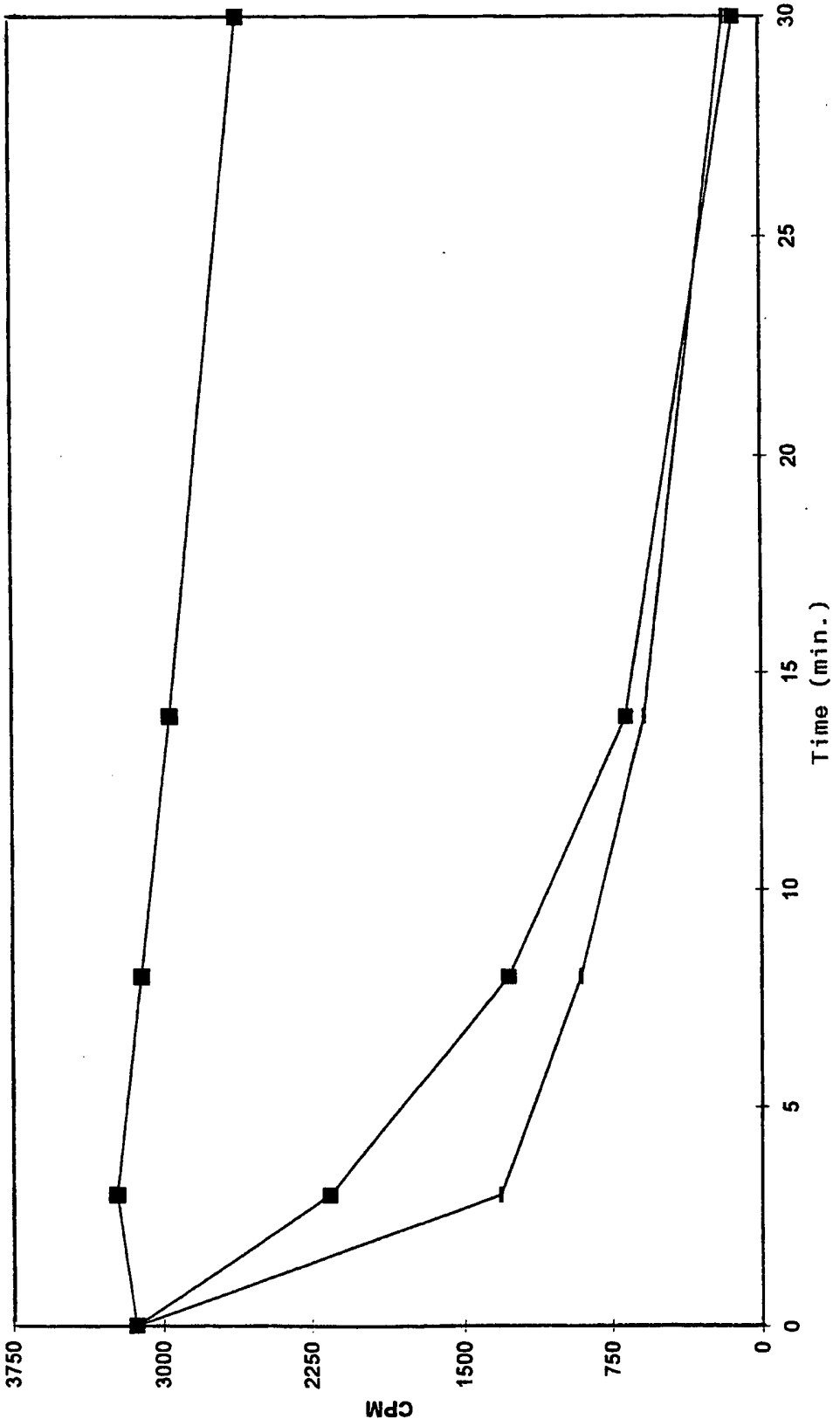


FIGURE 5B

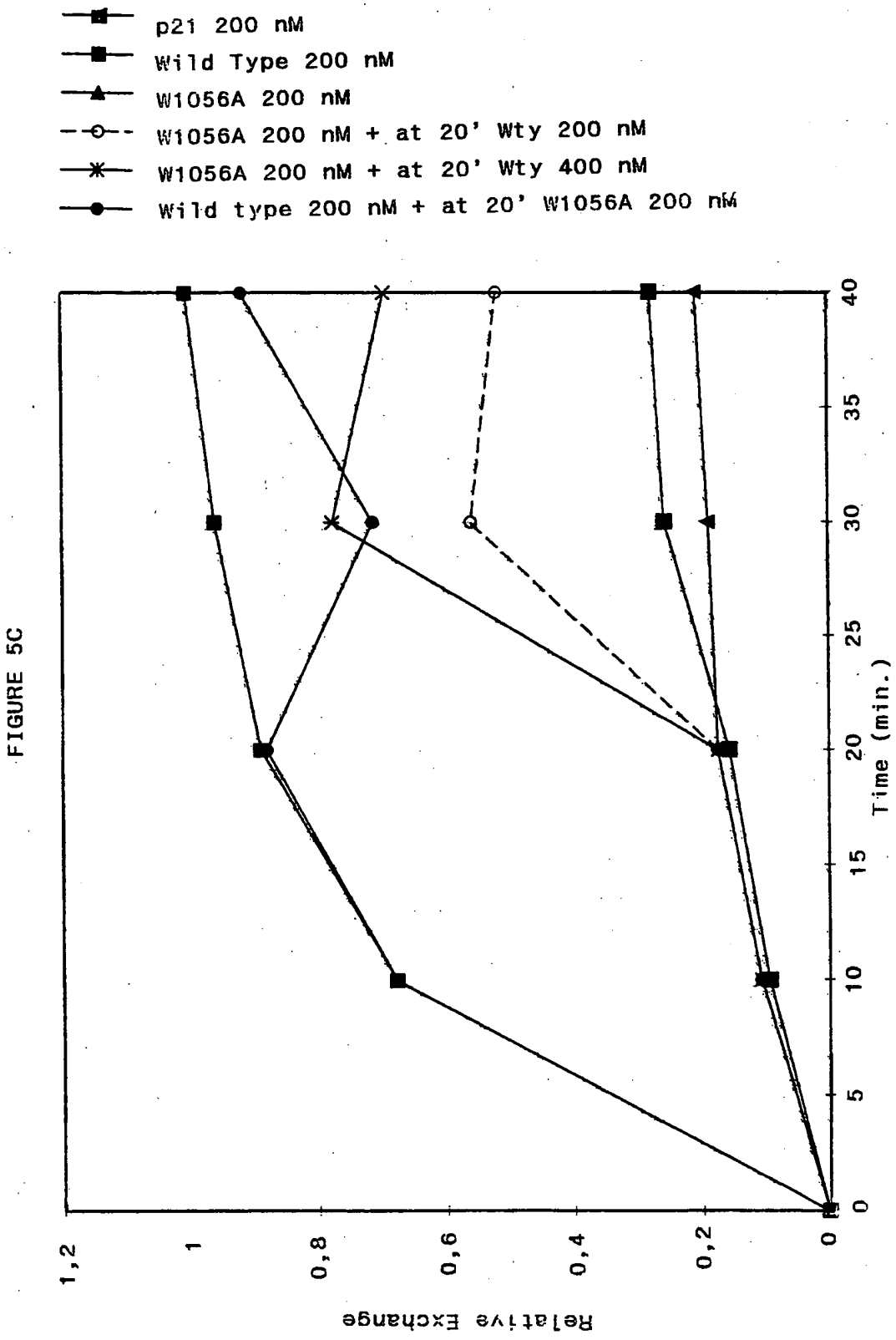

p21 200 nM
Wild Type 200 nM
W1056A 200 nM
W1056A 200 nM + at 20' Wty 200 nM
W1056A 200 nM + at 20' Wty 400 nM
Wild type 200 nM + at 20' W1056A 200 nM
Relative Exchange
1,2
1
0,8
0,6
0,4
0,2
0
Time (min.)
0
5
10
15
20
25
30
35
40

FIGURE 5C

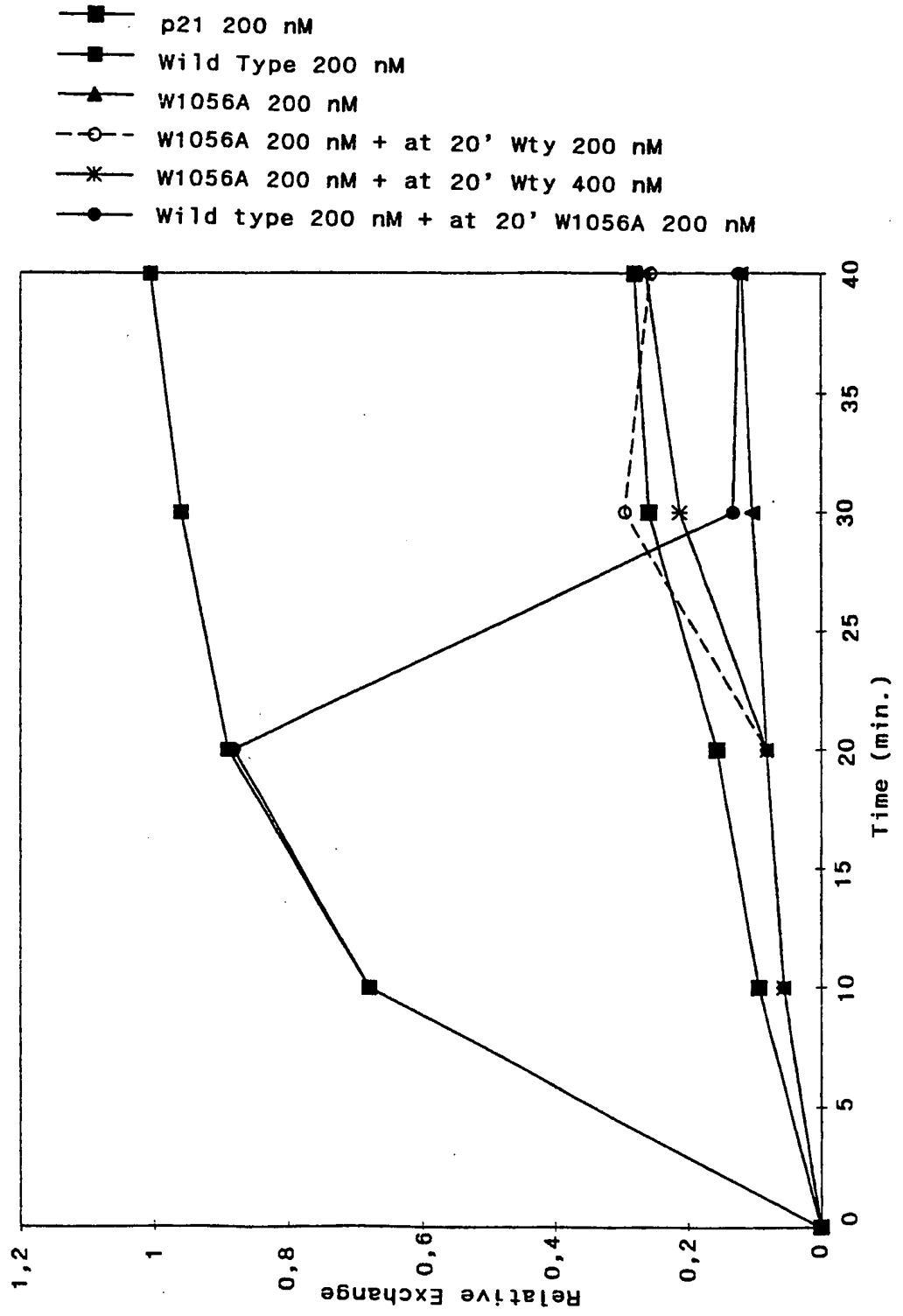

p21 200 nM
Wild Type 200 nM
W1056A 200 nM
W1056A 200 nM + at 20' Wty 200 nM
W1056A 200 nM + at 20' Wty 400 nM
Wild type 200 nM + at 20' W1056A 200 nM
Relative Exchange
1,2
1
0,8
0,6
0,4
0,2
0
Time (min.)
0
5
10
15
20
25
30
35
40

FIGURE 5D

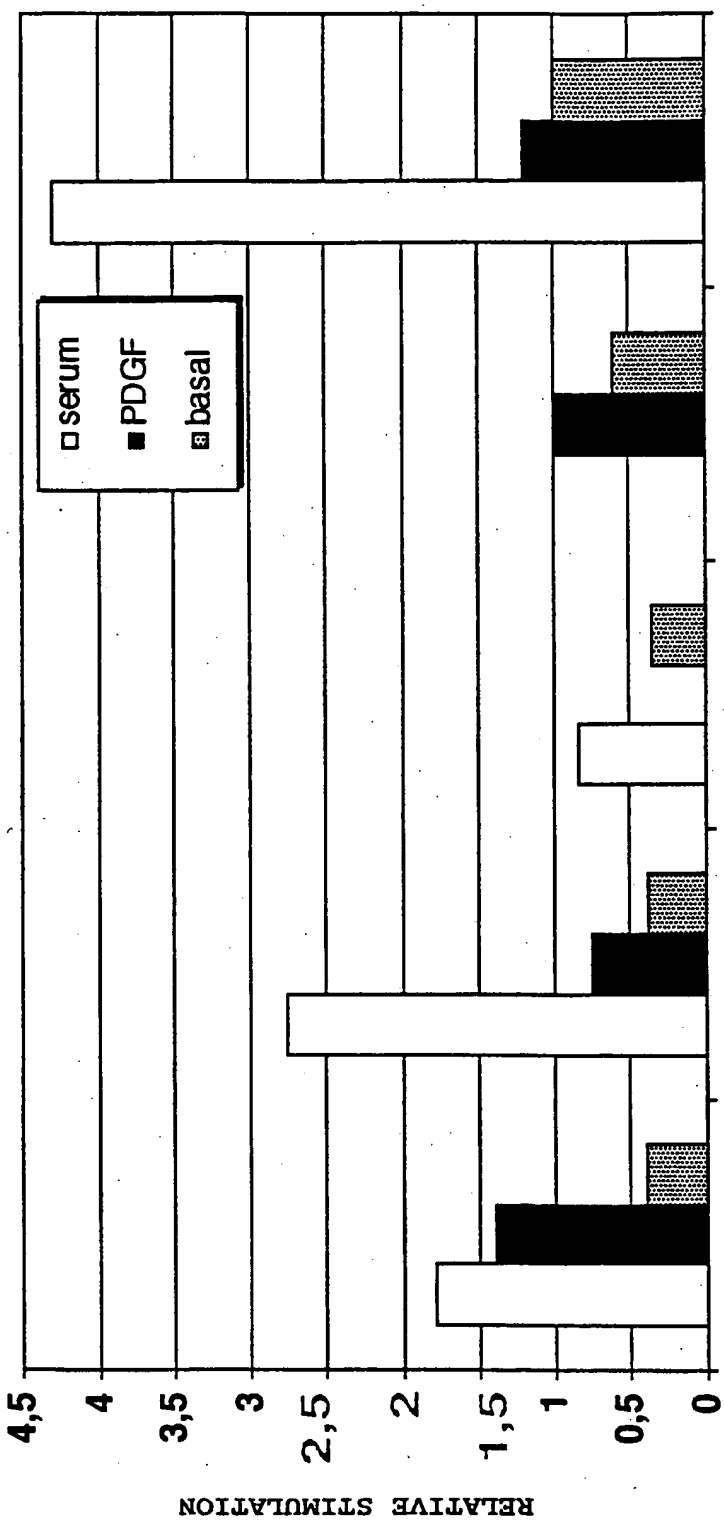

serum
PDGF
basal
wild type
WA
WE-SV
WE
pcDNA3
MUTANT
RELATIVE STIMULATION
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0

FIGURE 6

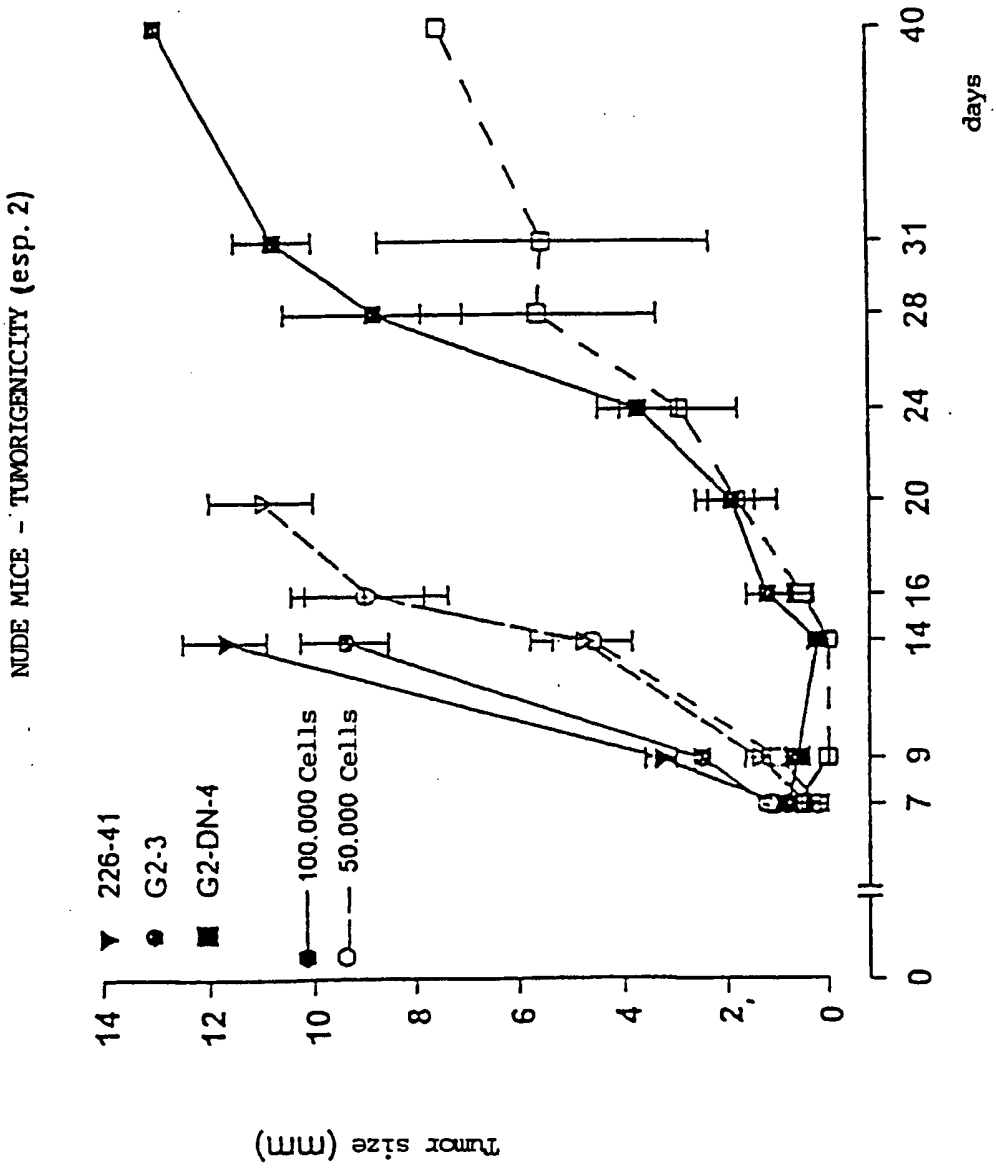

NUDE MICE - TUMORIGENICITY (esp. 2)
Tumor size (mm)
14
12
10
8
6
4
2
0
0
7 9
14 16
20
24
28
31
40
days
226-41
G2-3
G2-DN-4
100.000 Cells
50.000 Cells

FIGURE 7

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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8 July 1999 (08.07.99)

(54) Title: MUTANTS OF GEF PROTEINS

(57) Abstract

The present invention concerns a GEF mutant in which tryptophan (W) corresponding to position 1056 of the protein called $CDC25^{Mm}$ is mutated to an acidic amino acid, preferably glutammic acid, or tryptophan (W) at position 1056 and serine at position 1124 are mutated into an acidic acid and valine, respectively. The invention further provides the gene sequences encoding said amino acid sequences, and their application in the treatment of proliferative disorders and in the development of assays suitable for the identification of candidate agents able to disrupt the ras/GEF complex.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/04752

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/47 C07K19/00 G01N33/68 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PARK W. ET AL.: "Identification of a dominant-negative mutation in the yeast CDC25 guanine nucleotide exchange factor for Ras" ONCOGENE, vol. 14, no. 7, 20 February 1997, pages 831-836, XP002098824 see the whole document ---	1,11-13, 18
X	WO 93 21314 A (RHONE-POULENC RORER S.A. (FR); SCHWEIGHOFFER FABIEN; TOCQUE BRUNO) 28 October 1993 cited in the application see page 3, line 15-22 see page 7, line 24-28 see page 8, line 14-26 --- -/--	1,11-18

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
6 April 1999	16/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Macchia, G

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/04752

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	PARK W. ET AL.: "Amino acid residues in the CDC25 guanine nucleotide exchange factor critical for interaction with Ras" MOLECULAR AND CELLULAR BIOLOGY, vol. 14, no. 12, December 1994, pages 8117-8122, XP002098862 see abstract	
A	MARTEGANI E. ET AL.: "Cloning by functional complementation of a mouse cDNA encoding a homologoue of CDC25, a Saccharomyces cerevisiae RAS activator" EMBO JOURNAL, vol. 11, no. 6, June 1992, pages 2151-2157, XP000611568 cited in the application see page 2154; figure 2	

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 98/04752

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
See FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

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2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Due to the lack of data concerning the nucleotide / aminoacid sequences of members of the GEF class, other than CDC25Mm, the search of the subject matter of claims 2-10 was limited to mutants of CDC25Mm polypeptide, whose wild-type sequence is disclosed in sequence accession number P27671

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 98/04752

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9321314 A	28-10-1993	FR 2690162 A	22-10-1993
		CA 2131166 A	28-10-1993
		EP 0637334 A	08-02-1995
		JP 7505774 T	29-06-1995
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